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(57) Abstract

Micro–organisms having a chromosome in which at least one gene has been partly or wholly replaced by a homologous gene from another micro–organism, and an artificially introduced reporter gene is present and is expressed in a manner related to a homologous gene expression product. Panels of such micro–organisms are also described. Methods of assessing an agent for antibiotic activity and using the agent as an antibiotic. Methods of killing or inhibiting the growth of bacteria.

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BACILLUS STRAIN AND ASSAY METHOD

Whole-cell assays are known for specific inhibitors of *B. subtilis* proteins involved in chromosome partitioning and cell division. The property, of inhibiting chromosome partitioning and cell division, is indicative of actual or potential anti-microbial properties. The inventor has devised three such assays; they are described in WO 97/00325; WO 98/26087; and WO 98/26088, which are summarised below and to which reference is directed.

New compounds inhibitory for any chromosome partitioning and cell division functions are likely to have a broad spectrum of activity against a wide range of bacteria, including important pathogens, because the functions targeted appear to be highly conserved. However, it is possible that some of the compounds discovered may turn out to be relatively specific for the *B. subtilis* proteins, in which case they would not be useful general purpose antimicrobial agents.

A similar problem arises in any whole-cell assay for an inhibitor of a specific gene of any micro-organism. The problem is that an inhibitor of a specific gene of a particular strain or micro-organism, may be specific to that strain, or alternatively may have inhibitory properties which are exerted over a rather wide range of micro-organisms. The present invention addresses that problem by replacing a target gene in a micro-organism used for a whole-cell assay with a homologous gene from a different organism, e.g. a micro-organism of more direct interest.

Thus the invention provides in one aspect a micro-organism having a chromosome in which:

a) at least one gene has been partly or wholly replaced by a homologous gene from another micro-organism, and

b) an artificially introduced reporter gene is present and is

expressed in a manner related to a homologous gene expression product.

In another aspect the invention provides a method of assessing an agent for antibiotic activity, which method comprises incubating the micro-organism as defined in the presence of the agent, and observing expression of the reporter gene or genes.

The micro-organism may be for example a yeast or more preferably a bacterium. The bacterium may be a *Bacillus* species that is capable of growth and sporulation under suitable conditions and for which genetic constructs can be made. *B. subtilis* is conveniently accessible and well characterised and is preferred.

A homologous gene is a functionally equivalent gene from another micro-organism. In the micro-organism of the present invention, at least one gene (the target gene) has been partly or wholly replaced by a homologous gene from another micro-organism. Preferably the target gene is one which is well conserved over many different species of bacteria or other micro-organisms. It is necessary that the homologous gene be functionally incorporated so as to be capable of expression *in vivo*. When the target gene is partly or wholly replaced by a homologous gene, it is necessary that the homologous gene be capable of forming an expression product that is different in some respect from the expression product of the target gene. Suitable target genes include genes involved in DNA replication, RNA synthesis, protein synthesis, cell wall synthesis, transport and cell division.

For micro-organisms which are Bacillus species e.g. *B. subtilis*, cell division genes include *divIB* (also called *ftsQ*), *divIC, divIVA, ftsA, ftsL* (also called *mraR*), *ftsZ, pbpB*, as well as *spoOJ* and *spoIIIE*, and others, both known and to be discovered. Since these cell division genes are substantially conserved across many bacterial species, it is plausible that these engineered *Bacillus* strains will grow and sporulate with reasonable efficiency. The homologous gene may be taken from other bacilli or closely related organisms such as *clostridia* and *Listeria*. More

preferably, the homologous gene may be taken from a pathogenic bacterium such as staphylococci and streptococci. *B. subtilis* molecular genetic methods make it straightforward to replace any gene with a homologous gene from another bacterium.

An artificially introduced reporter gene is one which is not naturally present in the strain in question, and which may have been introduced by genetic manipulation. A reporter gene is one which on expression gives rise to an easily detected or observed phenotype. For example, the expressed protein may be an enzyme which acts on a substrate to give a product that is easily observed e.g. because it is coloured or chemiluminescent of fluorescent. Reporter genes capable of being expressed in *Bacillus* species and other micro-organisms are well known and documented in the literature. Reporter genes are preferably chosen so that their products can be readily assayed simultaneously. *lacZ* has been used for more than 10 years with great success in *B. subtilis* and there is a range of useful substrates that generate coloured or fluorescent products upon hydrolysis by ß-galactosidase. The *uidA* gene of *E. coli* has recently been harnessed for similar purposes, and the range of substrates available for the gene product, ß-glucoronidase is similar to that for ß-galactosidase.

In one example, two different fluorogenic substrates are used to assay the activities of the two reporters simultaneously in a single reaction.

On incubation of the micro-organism, e.g. on cell division or sporulation, a reporter gene is expressed in a manner related to the activity of an expression product e.g. a cell division protein, of the homologous gene. For example, decreased activity of that protein may be associated with either increased expression or reduced expression of the reporter gene. When two reporter genes are used, preferably expression of one is increased, and expression of the other is decreased, in association with a change in the level of activity of that protein.

The preferred assay method of the invention involves inducing the *Bacillus* strain described to sporulate in the presence of a putative anti-microbial agent. Preferably the *Bacillus* strain is contacted, just prior to asymmetric cell division with the agent. To screen agents on a large scale, samples of the *Bacillus* strain may be cultured in an exhaustion medium to stimulate sporulation; either in the wells of a microtitre plate to which the agent is added; or in bulk to be dispensed into the wells of a microtitre plate of which individual wells contain one or more different agents. After suitable incubation, observation is made of expression of the one or more reporter genes. For example, when the expression products of two reporter genes are different enzymes, substrates for the two enzymes may be added to the wells of the microtitre plate, and observation made of e.g. chemiluminescent or fluorescent or coloured products of enzymatic activity.

Use of such strains have several practical consequences:

i) It enables inhibitors which act on the protein product of a pathogen but not on that of a parent micro-organism e.g. *B. subtilis* to be identified.

ii) In the case of an assay for inhibitors of cell division, it may facilitate identification of the specific target of the inhibitor. By screening promising compounds against a series of strains in which cell division genes have been systematically replaced with homologues from other organisms, the specific target of the inhibitory compound becomes evident. Thus, for example, detection of a compound which inhibits the *B. subtilis* parent strain but not a derivative carrying the *S. aureus* homologue of *ftsZ*, would be strongly suggestive of a compound targeted on the FtsZ protein.

iii) A panel of strains with a given target gene systematically replaced by genes from other organisms also provides information about the spectrum of activity of each potential inhibitor. For example, some of the compounds found to inhibit the *B. subtilis* SpoIIIE protein might not act on the strain bearing its *S. aureus* homologue. Other compounds might

show non-species specific inhibition and act on a range of gene products from different organisms. Such tests provide a useful means of ensuring that new inhibitors have a broad spectrum of activities.

Thus in another aspect the invention provides a panel of the micro-organisms as defined, wherein in different members of the panel genes have been partly or wholly replaced by homologous genes from different micro-organisms. The invention also includes a method of assessing an agent for antibiotic activity, which method comprises incubating the members of the panel in the presence of the agent, and observing expression of the reporter gene or genes in different members of the panel. Also provided, is a method of killing or inhibiting the growth of bacteria, which method comprises contacting the bacteria with an agent which inhibits the growth of a micro-organism according to the invention.

According to **WO 97/00325**, a unique sporulation phenotype arising when *spoIIIE* is inactivated provides the potential for a very powerful and specific assay. In the absence of functional *spoIIIE*, the chromosome is trapped partially inside and partially outside the prespore compartment, but the prespore-specific transcription factor σ^F is activated normally. Reporter genes dependent on σ^F are expressed if they are located at certain places in the chromosome and blocked if they lie elsewhere. That invention provides a *Bacillus* strain having a chromosome with two reporter genes each linked to a promoter and responsive to the action of σ^F during sporulation, a first reporter gene being located in a segment of the DNA that is trapped in a prespore compartment when *spoIII* function is impaired, and a second reporter gene being located outside the said segment. An assay method using the *Bacillus* strain is also described.

The *B. subtilis spoIIIE* gene is required for translocation of the prespore chromosome through an asymmetrically positioned septum during sporulation in *B. subtilis*. Although at first sight this appears to be a very specialised mechanism, *spoIIIE*-like genes are highly conserved throughout bacteria. A more general function for the *B. subtilis* gene was revealed by

experiments in which wild type and *spoIIIE* mutant cells of *B. subtilis* were exposed to sub-lethal concentrations of inhibitors of DNA replication (Sharpe and Errington, 1995, Proc. Natl. Acad. Sci. USA **92**, 8630-8634). Under such conditions the probability of chromosomal DNA being caught in the division septum is increased. Wild type cells could recover from this state but in *spoIIIE* mutants the chromosome remained trapped and so these mutants were more sensitive to such inhibitors.

If removal of chromosomal DNA from the division septum is the general function of SpoIIIE protein, and its action during sporulation just an extreme manifestation of this function, the *spoIIIE*-like genes from non-sporulating bacteria might be able to functionally complement the defect of *spoIIIE* mutants of *B. subtilis* and restore their ability to sporulate.

According to the present invention, the *spoIIIE* gene is partly or wholly replaced by a homologous gene from another bacterium. The use of the homologous gene from *Streptococcus pneumoniae* is described in the example below.

According to **WO 98/26087**, the effects of *spoOJ* mutations on prespore chromosome orientation, and the ability to detect this by use of a *spoIIIE* mutant background, provides the potential for a very specific whole-cell assay for inhibitors of *spoOJ* function. The presence of any given segment of chromosomal DNA in the prespore can be detected by use of a reporter gene controlled by a transcription factor σ^F, which is activated only in the small prespore compartment (a process that is not affected by perturbations in chromosome partitioning).

WO 98/26087 thus provides a *Bacillus* strain having a chromosome with the following modifications:

a) a mutation of a *spoIIIE* gene which blocks transfer of the prespore chromosome,

b) a mutation in the *soj* gene which prevents loss of *spoOJ* function from blocking sporulation, together with

c) a first reporter gene having a promoter which is dependent on

σ^F factor and placed at a location where impaired *spoOJ* function leads to increased trapping and hence to increased expression from the prespore, and/or

d) a second reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired *spoOJ* gene function leads to reduced trapping and hence to reduced expression in the prespore.

The present invention provides a *Bacillus* strain of this kind in which the *spoOJ* gene has been replaced by its homologue from another bacterium.

Synthesis of σ factor begins at the onset of sporulation, but its product is initially held in an inactive state by the action of an anti-σ factor *spoIIAB*. Release from inhibition requires the concerted action of at least two other proteins, *SpoIIAA* and *SpoIIE*, which serve to allow release of σ^F activity only after the sporulating cell has undergone asymmetric cell division and to restrict the σ^F activity to the smaller prespore cell type. According to **WO 98/26088**, this dependence of σ^F activation on septation is used as the basis for a sensitive assay for inhibitors of cell division. Thus that specification provides a *Bacillus* strain having two reporter genes, a first reporter gene having a promoter which is dependent on active σ^F (or σ^E), and a second reporter gene having a promoter regulated similarly to the gene encoding the σ factor, to provide a measure of the synthesis of the (inactive) σ factor. A whole-cell screening method for identifying antimicrobial agents involves use of the *Bacillus* strain.

According to the present invention, any *Bacillus* cell division gene involved in these activities is partly or wholly replaced by a homologous gene from another bacterium. The *Bacillus* gene may be for example *divIB* (also called *ftsQ*), *divIC*, *divIVA*, *ftsA*, *ftsL* (also called *mraR*), *ftsZ*, *pbpB*, as well as *spoOJ* and *spoIIIE*.

Example

The inventors constructed a strain of *B. subtilis* in which the final 310 amino acid residues of the *spoIIIE* gene had been replaced with the equivalent section of the gene from *Streptococcus pneumoniae* strain R6, either in the correct or inverted orientation. In the correct orientation, the strain should make a hybrid protein comprising the poorly conserved membrane anchor region encoded by the *B. subtilis* gene fused to the highly conserved C-terminal coding region of the *Streptococcus pneumoniae* gene.

Strains with the *Streptococcus* gene inserted in either the correct or the inverted orientation, relative to the host *spoIIIE* gene, were induced to sporulate by a standard resuspension method, in parallel with an isogenic wild type strain. After 9 hours, the number of spores formed was measured on the basis of heat resistance, by heating at 80°C for 10 min and then plating serial dilutions on nutrient agar. Colonies were counted after overnight incubation. In the strain with the *Streptococcus* DNA inserted in the inverted orientation, and so with no intact *spoIIIE* gene, sporulation was completely abolished (< 10 heat resistant colony forming units [cfu] per ml of culture). However, with the *Streptococcus* DNA in the correct orientation, spore heat resistance was found to arise with approximately equal frequency (2.0×10^8 cfu per ml) to the wild type (1.3×10^8 cfu per ml). The outgrowth of a new colony from a heat resistant spore requires that the spore had acquired a complete chromosome. Thus, the hybrid gene must have been able to catalyse chromosome transfer into the spore compartment just as well as the wild type SpoIIIE protein.

CLAIMS

1. A micro-organism having a chromosome in which:

a) at least one gene has been partly or wholly replaced by a homologous gene from another micro-organism, and

b) an artificially introduced reporter gene is present and is expressed in a manner related to a homologous gene expression product.

2. The micro-organism of claim 1, wherein the gene is involved in DNA replication, RNA synthesis, protein synthesis, cell wall synthesis, transport or cell division.

3. The micro-organism of claim 1 or claim 2, which is a bacterium.

4. The micro-organism of claim 3, wherein the bacterium is a *Bacillus* strain capable of growth and sporulation and in which at least one gene has been partly or wholly replaced by a homologous gene from another bacterium.

5. The *Bacillus* strain of claim 4, wherein:

a) a *spoIIIE* gene has been replaced by its homologue from another bacterium, and

b) two reporter genes are present each linked to a promoter and responsive to the action of σ^F during sporulation, a first reporter gene being located in a segment of the DNA that is trapped in a prespore compartment when SpoIIIE function is impaired, and a second reporter gene being located outside the said segment.

6. The *Bacillus* strain of claim 5, wherein a *spoIIIE* gene has been partly or wholly replaced by a homologous gene from *Streptococcus pneumoniae.*

7. The *Bacillus* strain of claim 4, wherein:

a) a cell division gene has been partly or wholly replaced by its homologue from another bacterium, and

b) two artificially introduced reporter genes are present, a first reporter gene having a promoter which is dependent on active σ^F or σ^E factors, and a second reporter gene which provides a measure of the synthesis of the (inactive) σ^F or σ^E factor.

8. The *Bacillus* strain of claim 4, wherein the strain is modified by a mutation of a *spoIIIE* gene which blocks transfer of the prespore chromosome, and:

a) a *spoOJ* gene has been replaced by its homologue from another bacterium, and

b) one or two reporter genes are present, a first reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired S*poOJ* function leads to increased trapping and hence to increased expression in the prespore, and/or a second reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired *SpoOJ* function leads to reduced trapping and hence to reduced expression in the prespore.

9. The *Bacillus* strain of any one of claims 4 to 8, which is a *B. subtilis* strain.

10. A method of assessing an agent for antibiotic activity, which method comprises incubating the micro-organism of any one of claims 1 to 4 in the presence of the agent, and observing expression of the reporter gene or genes.

11. The method of claim 10, wherein the *Bacillus* strain of any one of claims 4 to 9 is induced to sporulate in the present of the agent.

12. A method of determining whether an agent inhibits SpoIIIE function in *Bacillus* species, which method comprises inducing the *Bacillus* strain of claim 5 or claim 6, to sporulate in the presence of the agent, and observing expression of the first and the second reporter genes.

13. A method of determining whether an agent inhibits cell division in *Bacillus* species, which method comprises inducing the *Bacillus* strain of claim 7 to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and second reporter genes.

14. A method of determining whether an agent inhibits SpoOJ function in *Bacillus* species, which method comprises inducing the *Bacillus* strain of claim 8 to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and/or the second reporter gene.

15. The method of any one of claims 11 to 14, wherein the *Bacillus* strain is induced to sporulate and is contacted, just prior to asymmetric cell division, with the agent.

16. A panel of the micro-organisms of any one of claims 1 to 9, wherein in different members of the panel genes have been partly or wholly replaced by homologous genes from different micro-organisms.

17. A method of assessing an agent for antibiotic activity, which method comprises incubation of the members of the panel of claim 16 in the presence of the agent, and observing expression of the reporter gene or genes in different members of the panel.

18. A method which comprises incubating a micro-organism of any one of claims 1 to 9 in the presence of an agent, observing expression of the one or more reporter genes and thereby determining that the agent inhibits the growth of the micro-organisms, and using the agent as an antibiotic.

19. A method of killing or inhibiting the growth of bacteria, which method comprises contacting the bacteria with an agent which inhibits the growth of a micro-organism of any one of claims 1 to 9.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/18 C12Q1/68 C12N15/75 C07K14/32 C12R1/07

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N C07K C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 05244 A (UNIV DUKE) 2 April 1992 (1992-04-02) the whole document	1,2,10, 16,17
P,X	WO 99 18211 A (BENEGAL ANUPAMA N ;CADUS PHARMACEUTICAL CORP (US); KLEIN CHRISTINE) 15 April 1999 (1999-04-15) the whole document	1,2,10, 16,17
A	WO 97 00325 A (ISIS INNOVATION ;ERRINGTON JEFFERY (GB)) 3 January 1997 (1997-01-03) cited in the application the whole document	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 26087 A (ERRINGTON JEFFERY ;ISIS INNOVATION (GB); WU LING JUAN (GB)) 18 June 1998 (1998-06-18) cited in the application the whole document	
A	WO 98 26088 A (ERRINGTON JEFFERY ;ISIS INNOVATION (GB)) 18 June 1998 (1998-06-18) cited in the application the whole document	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
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